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Botulism due to food toxin is caused mainly by seven known serotypes of protein neurotoxins, botulinum neurotoxins (BoNt) produced by *clostridium botulinum*. BoNts are the most potent toxins and poisons known. The purpose of this work is to design a synthetic peptides vaccine for protection against BoNt. Regions of BoNt/A will be selected for synthesis, based on a number of sequence and conformational parameters and their immunological activities towards anti-BoNt/A antibodies and T cells determined. The immunodominant epitopes will be used as immunogens and those peptides which stimulate BoNt/A-cross reactive antibody and/or T cell responses will then be investigated for their capacity to provide active and passive immunity against BoNt/A challenge. The epitopes that are most protective when employed individually as immunogens will be used, in a mixture and in a multiepitope-carrier conjugate, as a vaccine for both active and passive protection of mice. The synthetic vaccine against BoNt/A will be used as a prototype to prepare synthetic vaccines containing the regions of the other serotypes that are counterparts to the regions found to be most protective on BoNt/A.

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M Zouhair Attaj April 10, 1995

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INTRODUCTION

Botulism due to toxin in food is caused mainly by a group of protein neurotoxins, botulinum neurotoxins (BoNt) produced by *clostridium botulinum* (Brooks, 1956). Botulinum neurotoxins are the most potent toxins and poisons known (Llamanna, 1959; Middlebrook, 1989). Poisoning can occur by ingestion of clostridia contaminated food (foodborne botulism), by infant bowel infection (infant botulism) and by infection of wounds (wound botulism) (Sakaguchi *et al.*, 1988; Tacket and Rogawski, 1989).

Seven immunological serotypes of BoNt (A through G) are known of which type C has two subtypes (C1 and C2) (Simpson, 1979, 1981). *C. botulinum* shows widespread distribution in soil in the United States. Type A predominates in the Western states and type B in the east (Smith, 1977; Hatheway, 1989). Human botulism is most frequently caused by types A, B and E and rarely by type F, while animals are more often infected by types C and D (Sakaguchi *et al.*, 1988).

Botulinum neurotoxins are synthesized as a single chain, or progenitor toxin, (Beers and Reich, 1969; Ohishi and Sakaguchi, 1975) of a molecular weight of 150-170 KD (Simpson, 1979). They are activated by a proteolytic enzyme which causes a scission (nicking) at a single polypeptide chain. In clostridia strains producing BoNt types A, C, D and some types of B and F, the proteolytic enzyme is endogenous, while other strains (type E and some types B and F) must rely on an exogenous protease (e.g. trypsin) for activation (Das Gupta and Sugiyama, 1972; Beers and Reich, 1969; Simpson, 1979). The active forms of the various types of BoNt appear to have a common subunit structure (Das Gupta and Sugiyama, 1972, Beers and Reich, 1969; Sugui and Sakaguchi, 1975; Yang and Sugiyama, 1975, Miyazaki *et al.*, 1977; Syuto and Kubo, 1979; Iwasaki *et al.*, 1980; Ohishi *et al.*, 1980; Kozaki *et al.*, 1981). Typically, the two subunits resulting from the nicking of the progenitor BoNt have molecular weights of about 100 KD (heavy or H chain) and 50 KD (light or L chain). Except in BoNt type C2, the two subunits are held together by a disulfide bond (Das Gupta and Sugiyama, 1972; Yang and Sugiyama, 1975; Miyazaki *et al.*, 1977; Kozaki *et al.*, 1981).

BoNt induces a blockade of neuromuscular transmission (Simpson, 1986, 1989) in a manner similar to that previously described for diphtheria toxin (Middlebrook and Dorland, 1977, 1979; Middlebrook *et al.*, 1978, 1979; Middlebrook, 1981). The first step in the paralysis involves binding of BoNt to an acceptor molecule on the cell surface. The binding step does not seem to have an effect on cell function. In the second step, the toxin-receptor complex undergoes endocytosis and, in the third step, the internalized toxin blocks neurotransmitter release. Although this mode of action appears to be applicable to many other bacterial and plant toxins (Neville and Chang, 1978; Schmitt *et al.*, 1981; Simpson, 1986), the molecules on the membrane and inside the cell which are involved in these interactions and activities are not well understood.

Although it is generally accepted that toxin endocytosis is receptor mediated, the exact nature of the receptor molecule(s) remains perhaps unknown. Over 30 years ago, there was some excitement that gangliosides might be at least part of the receptor to

which BoNt binds (Simpson and Rapport, 1971). Indeed it was found that ganglioside GT1b is effective at inactivating BoNt (Kitamura *et al.*, 1980). Different BoNt serotypes do not share a common receptor (Kozaki, 1979). BoNt/A, B, E and F were markedly inactivated, while BoNt types C and D suffered only mild inactivation by ganglioside GT1b (Kozaki *et al.*, 1984). But although gangliosides do bind to BoNt, the studies are usually done under non-physiological conditions of pH and ionic strength and there has been no evidence showing that the binding of BoNt to gangliosides is related to its effect on the cell (Middlebrook, 1989). Binding of gangliosides to BoNt may take place at a site that is different from its binding site to the receptor. Interference of gangliosides with BoNt binding to receptor may be due to an allosteric or a conformational change in BoNt, resulting in alteration of its receptor-binding site in a similar way that certain nucleotides inhibit the binding of diphtheria toxin to its receptor (Middlebrook and Dorland, 1979; Middlebrook *et al.*, 1978; Eidels *et al.*, 1983).

Extensive studies have indicated that BoNt binds to synaptosomes, that different BoNt types bind to different acceptors and that BoNt undergoes acceptor-mediated endocytosis (Donavan and Middlebrook, 1986; Black and Dolly, 1986). However, evidence is still lacking that binding to synaptosomes has physiological relevance (Middlebrook, 1989). It was recently shown with TeNt that binding to synaptosomes may not be relevant to toxicity (Bakry *et al.*, 1991). Even when BoNt is injected directly into the brain, it does not appear to have any effect on the central nervous system. It is well established that the H chain binds to the acceptor thereby allowing the L chain, or a combination of H and L chains, to be internalized and cause paralysis. There is some evidence (Poulain *et al.*, 1989a,b) that the C-terminal half of the H chain together with the L chain are active intraneuronally. Interestingly, the C-terminal fragment of the TetTX H chain does not show this activity.

It has long been recognized that BoNt acts upon the nervous system (Dickson and Shevky, 1923a, b) and causes paralysis. The mechanism is not well delineated but it has been established that paralysis is due to blockage of acetylcholine (ACh) release from nerve terminals in the neuromuscular junction (Burgen *et al.*, 1949; Wright, 1955; Brooks, 1956; Simpson, 1980, 1981; Gunderson, 1980). Because the exact mechanism of paralysis caused by BoNt is not known, potential therapeutic drugs cannot be based on rational design. This is further complicated by the findings that the mechanism by which different BoNt serotypes cause paralysis are not exactly the same. Therefore, the design and preparation of effective drug antagonists is not likely to be achieved at the present time (Thesleff, 1989).

An immunological approach provides an alternative and more effective means for protection against BoNt. For use as an antigen, BoNt is usually treated for about 7 days with formaldehyde (which renders it non-toxic) and injected in horse. Timely administration of this antitoxin may reduce morbidity and mortality (Tacket and Rogawski, 1989). Protection by passive immunity requires proper diagnosis and the rapid access to an antitoxin. Because the latter is not always possible, active immunization will obviously offer a permanent and more secure protection. Toxoids that are prepared by formaldehyde detoxification may not be the best antigens for this purpose. Reversion to toxicity might occur on standing and this has been reported for tetanous and diphtheria

toxoids (Akama *et al.*, 1971a,b). Although, this might be minimized or overcome by storing the toxoid in formaldehyde, such a prolonged exposure causes drastic chemical and immunological changes in proteins (Atassi, 1977). Antibodies against both H and L chains of BoNt types B and E showed neutralizing activity (Kozaki *et al.*, 1977, 1989). Some mAbs against the H chain of BoNt/E possessed neutralizing activity (Kozaki *et al.*, 1986, 1989; Simpson *et al.*, 1990). Neutralizing mAbs vary in their sensitivity to conformational changes in BoNt (Niemann, 1991). The complete primary structures of BoNt/A (Binz *et al.*, 1990a), C1 (Hauser *et al.*, 1990) and D (Binz *et al.*, 1990b) and partial sequences of types B (Niemann, 1991) and E (Binz *et al.*, 1990a; Das Gupta and Foley, 1989) have been determined. BoNt shows extensive homology to TeNt (Eisel *et al.*, 1986; Binz *et al.*, 1990a,b; Niemann, 1991). Four cysteine residues are conserved in BoNt/A and TeNt. The disulfide bridges and the position of the sulfhydryl groups were recently determined in TeNt (Krieglstein *et al.*, 1990). Crystallization and preliminary X-ray analysis of BoNt type A were recently reported (Stevens *et al.*, 1991). Therefore, adequate structural information is now available for the design of a vaccine based on a rational molecular strategy.

The purpose of this proposed research is to develop a peptide-based strategy for protection against botulinum neurotoxin. The strategy is based on the identification and synthesis of appropriate regions of the neurotoxin. These synthetic peptides will then be used as immunogens to stimulate active, or passive (by antibody transfer) immunity against toxin poisoning. Of course, in order for the approach to be successful, it is crucial that the appropriate regions on the protein toxin be correctly identified, designed and synthesized. The peptides can then be employed in a suitable active immunization strategy to stimulate protection in the host. Alternatively, antibodies (polyclonal or monoclonal) are first made against the peptides in an animal host and then used to confer, by transfer, passive protection in recipient human individuals.

Recent studies (Middlebrook, 1995) have shown that immunization with a C-terminal fragment (C-fragment, resides 860-1295) of BoNt/A afforded excellent protection against BoNt/A poisoning. The studies described here were, therefore, carried out to map the antibody and T-cell epitopes of the C-fragment and then to subsequently evaluate in mice the protective capability against BoNt/A challenge, of the synthetic epitopes, individually and in appropriate combinations when they are used as immunogens. The results reported in this Midterm Progress Report describe the mapping of the antibody epitopes recognized by human and horse antisera against BoNt/A and the T-cell epitopes recognized by two different mouse strains after immunization with the C-fragment.

METHODS

Synthesis and Purification of the Peptides

The overlapping peptide spanning the entire C-fragment (residues 855-1295) (Figure 1) were prepared by Fmoc chemistry by the procedure recently described in detail (Atassi, *et al.*, 1991). Solid phase peptide synthesis was done on a benzyloxybenzyl alcohol resin (Vega Biotechnologies) to which 9-fluorenylmethylcarbonyl (Fmoc)-glycine

had been coupled. Removal of the N^{α}-Fmoc group before each coupling was done by treatment of the peptide resin with 20% piperidine in dimethylformamide (DMF) for 10 min. This was followed by washing (3 times each, 30 sec) with DMF, methanol, and then DMF. Coupling of consecutive amino acids was done for 2 hr by using 3-molar excess of each of the Fmoc amino acid derivatives, diisopropylcarbodiimide in DMF/CH_2Cl_2, 1:1 (vol/vol) and 1-hydroxybenzotriazole. The resin was then washed with DMF and methanol (three times each, 30 sec), followed by two 30-sec washes of CH_2Cl_2. The completion of coupling after each residue was monitored by ninhydrin, and recoupling was repeated when necessary. After the last cycle and deprotection of the Fmoc-group, the peptide was cleaved from the resin by treatment (2.5 hr) with 55% trifluoroacetic acid in CH_2C1_2, and the solvent removed on a rotary evaporator. The peptide was washed three times with cold ether, dissolved in water, and freeze-dried. The products were purified by chromatography on CM-Sephadex C50 or DEAE-Sephadex A50, as appropriate. The homogeneity of the peptides was checked by high-voltage paper electrophoresis and by analytical HPLC on a C_{18} column using a gradient of 0.1% trifluoracetic acid in water/0.1% trifluoracetic acid in acetonitrile. The amino acid compositions of the peptides were also determined.

Analytical Methods

Completed synthetic peptides, partial synthetic products at selected states during synthesis and conjugates to carriers were checked by amino acid analysis. For amino acid analysis, proteins or peptide samples (0.1 mg) dissolved in 0.5 ml of constant boiling HC1 (double distilled) flushed with nitrogen, evacuated, sealed, heated at 110° C for 22, 48 or 72 hours. The hydrolysates were then be freed of excess HC1 on a rotary evaporator. Tryptophan was determined by amino acid analysis of hydrolysates with p-toluenesulfonic acid containing 0.2% 3-(2 aminoethyl) indole (Liu and Chang, 1971). The hydrolysates were then assayed on a Beckman model 6300 amino acid analyzer. The values of serine and threonine) were obtained by extrapolation to zero hydrolysis time. Purity of synthetic peptides was checked by high voltage (3000 volt) paper, electrophoresis in pyridine-acetate at pH 3.65, and by chromatography on an analytical Vydac C18 HPLC column in o.1% trifluoroacetic acid-acetonitrile gradient.

Antisera

Human and horse antisera against inactivated BoNt/A were obtained from Dr. John L. Middlebrook (Fort Detrick, Frederick, MD)

Radiolabelling of Antibodies and Protein A

Antibodies and purified protein A (Pharmacia) were radiolabeled with ^{125}I (Amersham Corp., Arlington Heights, IL) using the chloramine-T method. Unbound ^{125}I was separated from the radiolabeled protein A by gel filtration on Sephadex G-25 (Pharmacia). At least 95% of the protein A-associated ^{125}I was precipitable with 10% (v/v) trichloroacetic acid).

Binding of anti-BoNt/A antibodies to the overlapping peptides

Binding of anti-BoNt/A antibodies to the synthetic peptides was determined by a solid-phase (plate) radioimmune assay (RIA). Briefly, flexible polyvinylchloride 96-well

plates (Becton Dickinson-Falcon, CA) were coated in triplicates or duplicates (3h, 37°C, then overnight at room temperature) with the overlapping peptides [2.5 μg in 50 μl of 0.15M NaCl in 0.01M sodium phosphate buffer, pH 7.2 (PBS) per well] or C-fragment (1 μg/50 μl PBS/well). Ovalbumin and bovine serum albumin (BSA)(both from Sigma Chemicals, MO) and an unrelated synthetic peptide were used as negative controls. After coating, the wells were washed five times with PBS, blocked with 0.5 % BSA in PBS (100 μ1, lh, 37°C) to prevent non-specific binding in subsequent steps and washed (5 times) with PBS. Aliquots (50 μl) of antisera (prediluted appropriately with PBS-0.1% BSA) were added to the wells, and the plates were incubated overnight at 4° C. The wells were washed with PBS, and incubated (2h, 37°C) with 50 μl of affinity purified appropriate 2nd antibodies that had been diluted with PBS-0.1% BSA. After washing with PBS, ^{125}I-labeled protein A was added to the wells ($2x10^5$ cpm in 50 μl of PBS-0. 1% BSA/well). The plates were incubated for 2h at room temperature, washed and the wells were cut out and the bound radioactivity counted on a gamma counter (1227 Gammamaster, LKB, Finland). The results were expressed as net cpm in which the average prebleed value of cpm bound to the same antigen was subtracted. For the titration of antisera, aliquots (50 μ1) of serial dilution (with PBS-0.1% BSA) of serum sample were added to the C-fragment- or selected peptide-coated wells.

Immunization of mice for T cell studies

Two strains of mice, SJL/JCr (H-2^s) and Balb/c (H-2^d) were used in these studies. Seven to eight week old female mice (National Cancer Institute, MD) were immunized (10 mice per strain) subcutaneously at the base of the tail with C-fragment (0.25 μg/mouse) in an emulsion (100 μl) of equal volumes of the C-fragment in PBS and CFA containing *Mycobacterium tuberculosis,* strain H37Ra (Difco Laboratories, MI).

Lymphocyte proliferation assay

Inguinal and paraaortic lymph node cells (LNC) were harvested and pooled from C-fragment-primed SJL and Balb/c mice 7-8 days after the immunization. Cells were co-cultured in triplicates in flat bottom microtiter plates (Corning, NY) at $5x\ 10^5$ cells/well with various concentrations of BoNt/A peptides (0.6-80 μg/ml), C-fragment (0.06-5 μ g/ml), Con A (1 μg/ml), LPS (500 μg/ml), control unrelated proteins (ovalbumin, l00 μ g/ml; lysozyme, 100 μg/ml; myoglobin, 100 μg/ml) or an unrelated synthetic peptide (sequence: ESSGTGIESSGTGI, 10-40 μg/ml) in a final volume of 200 μl of RPMI 1640 medium (Gibco, NY) supplemented with 2 mM L-glutamine, 10 mM HEPES, $5x10^{-5}$ M 2-mercaptoethanol, 100 U/ml penicillin, l00 μg/ml streptomycin containing 1% normal mouse serum. After 3 days of incubation at 37°C in a humidified, 5% CO_2 atmosphere, the cultures were pulsed with ^{3}H-thymidine (2 μCi/well, Research Products International, IL) and then harvested for counting by liquid scintillation. Results were expressed as stimulation index (S.I. = mean cpm incorporated by stimulated cells/mean cpm incorporated by unstimulated cells). For the purpose of this study, an S.I. value >2 was regarded as a positive response. Only one cell concentration ($5xlO^5$/well) was employed in this experiment.

RESULTS

Synthesis and characterization of the peptides

Thirty-one consecutive overlapping peptides were synthesized for the present work. The primary structures of the synthetic peptides are shown in Figure 1. The peptides, which were 19 residues each (except for peptide 31 which was 21 residues) and overlapped by 5-residues, spanned the entire polypeptide chain of the C-fragment (residues 855-1295). After purification, each of the synthetic peptides was homogeneous by analytical HPLC and its amino acid composition was in excellent agreement with that expected from its structure (Figure 1).

Initially, only the first 18 peptides were made. The remainder of the C-fragment, (i.e. region 1107-1295), were covered by 11 petides supplied to us by Fort Detrick, which they had made commercially. However, upon careful analysis, we found that these peptides had serious deletions in their structures and after consultation with Dr. Middlebrook, it was agreed that resynthesis of the region 1107-1295 was badly needed. We did this unexpected work and it was the cause of some delay and cost. At any rate, we caught up and the complete panel of the peptides has proved to be very effective in mapping the immunological recognition of the C-fragment.

Mapping of the regions on the C-fragment which bind human anti-BoNt/A antibodies

The human antiserum against BoNt/A was obtained from Dr. John L. Middlebrook (Fort Detrick, Fredrick, MD). It is an 1gG fraction of a pool of antisera from several volunteers who were injected with formaldehyde-inactivated BoNt/A. The antiserum was tested at dilutions of 1:1000 and 1:2000 vol/vol in 0.1% BSA in PBS. The results of antibody binding at these two dilutions to the synthetic overlapping peptides of BoNt/Aand the C-fragment are summarized in Figure 2. High amounts of antibodies were bound by peptides 2 (residues 869-887), 6 (residues 925-943), 10 (residues 981-999) at 1:1000 dilution, 11 (residues 995-1013), 15 (residues 1051-1069) and 24 (residues 1177-1195). In addition, low amounts of antibodies were bound by peptides 5 (residues 911-929) at 1:1000 dilution, 7 (residues 939-957), 9 (residues 967-985), 20 and 21 (residues 1121-1139/1135-1153 overlap and 29/30/31 (residues 1247-1265/1261-1279/1275-1295) overlap. Peptides 2, 6, 11, 15 and 24 exhibited high binding at both dilutions. On the other hand, peptide 10 which bound high amounts of antibodies at 1:1000 dilution showed little binding when the serum was diluted to 1:2000. It appears, therefore, that the antibodies against the peptides 9/10/11 overlap are of lower affinity than the antibodies directed against peptides 2, 6, 15 and 24.

Binding of horse anti-BoNt/A antiserum to BoNt/A peptides

Horse anti-BoNt/A antiserum was obtained from Dr. John L. Middlebrook (Fort Detrick, Fredrick, MD). The antiserum was tested at several dilutions and we found that it was not very strong. Figure 3 summarizes the binding results at 1:250 serum dilution with overlapping synthetic peptides and the C-fragment of BoNt/A. Perhaps because of the low, antibody titer in the antiserum, the areas that gave positive binding were very limited. These were (in the order of decreased binding): Peptides 31, 1, 30, 25 and 7. In

addition, three or four other peptides (3, 5, 11, 22 and 26) gave low but significant and reproducible binding.

T-Cell recognition of the C-fragment and its synthetic peptides

The lymphocyte proliferative studies, after immunization with the C-fragment, were carried out in two mouse strains. Balb/c and SJL

1. ***Reponses of Balb/c T-cells.*** The results are tabulated in Table 1 which gives the cpm values incorporated by the cells *in vitro* in response to the optimum challenge dose of each of the thirtyone peptides. The table also gives the label incorporated when the cells were not challenged (media values) or when they were challenged with Con A, lipopolysaccharide (LPS) (positive controls), lysozyme, myoglobin and ovalbumin (negative controls). The proliferation results are also expressed in stimulation index [SI=cpm incorporated by antigen challenged cells/cpm incorporated by unstimulated cells (media)]. The results in net cpm and in SI are summarized in Figures 4 and 5. Lymph node cells from Balb/c mice that had been primed with the C-fragment proliferated *in vitro* to challenge with the C-fragment or with peptides 21, 7 and 12. Lower but significant responses were obtained in response to challenge with peptides 8, 13, or 19. The cells were viable and responded strongly to Con A and LPS but did not respond to unrelated proteins.

2. ***Responses of SJL T cells.*** The results with LNS from SJL mice that had been primed with the C-fragment are given in Table 2 in total cpm, net cpm, [corrected for cpm incorporated by unstimulated cells (media)] and SI values, at optimum challenge dose of each of the thirty-one overlapping synthetic peptides and the C-fragment. Figure 6 and 7 show the results graphically in net cpm and SI values, respectively. The SJL mouse strain was very high responder to the C-fragment. The cells responded very strongly (SI=20-48) to peptides 4, 5, 6, 7, 8 and 9. They also gave a strong response (SI=9.9) to peptide 15. The cells were viable and responded to Con A and LPS and showed no significant responses to challenge with unrelated proteins and peptide.

DISCUSSION

The recent findings (Middlebrook, 1995) that immunization of mice with the C-fragment of BoNt/A afforded protection against toxin challenge indicated that the immunological mapping of this region of BoNt/A would be extremely valuable for the eventual design of a synthetic peptide vaccine against BoNt. Although the horse antiserum did not have a high titer, it was possible to determine regions capable of antibody binding. Several regions of the C-fragment were recognized by human and horse anti-BoNt/A antibodies. In T cell studies, Balb/c ($H\text{-}2^d$) was a moderate responder while SJL ($H\text{-}2^s$) was a very high responder to the C-fragment. In Balb/c, three regions were recognized by C-fragment-primed T cells. These were within peptides 7/8 overlap, 12/13 overlap, and 21. The regions recognized strongly by T cells from C-fragment-primed SJL mice concentrated within the large area spanned by peptides 4, 5, 6, 7, 8 and 9 in the first N-terminal third of the C-fragment. There was only one additional region within peptide 15 which stimulated a moderate response in these T cells. The concentration of the T cell

epitopes recognized by SJL T cells to the first third of the C-fragment is unusual and its significance (in terms of protection) in this strain will be the subject of further investigation. It is necessary for a successful design of a synthetic vaccine against a protein neurotoxin to take into account both antibody and T cell recognition epitopes. Thus, having determined in humans the antibody recognition epitopes on the C-fragment, it will now be valuable to determine the T cell recognition epitopes of a few human donors. For studies in mice, it will now be necessary to determine the antibody recognition epitopes in Balb/c and SJL. This will enable comparison of the antibody and T cell recognition profiles in the same mouse strain. With the knowledge of the antibody and T cell recognition epitopes in humans and mice, we will design and prepare multi-epitope synthetic vaccines.

CONCLUSIONS

In the first half of this contract support, we have mapped in human and horse anti-BoNt/A antibodies the recognition epitopes on the protective C-fragment (residues 855-1295) of the BoNt/A molecule. Using two mouse strains [Balb/c ($H\text{-}2^d$) and SJL ($H\text{-}2^s$)], the epitopes on the C-fragment recongnized by C-fragment primed T-lymphocytes were mapped. Identifcation of the antibody and T-cell recognition profiles is an important first step in the intricate requirments for the design of a synthetic vaccine.

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TABLE 1

Response to BoNt/A peptides 1-31 of LNC from **Balb/c** mice primed with 0.25 μg/mouse of C-Fragment of Botulinum Toxin

Challenge Antigen		CPM ± SD	Net CPM	Opt. Dose (μg/ml)	SI ± SD
Media		3534 ± 141	0		1.00 ± 0.04
Con A		51281 ± 2678	47747		14.51 ± 0.76
LPS		47271 ± 5254	43737		13.39 ± 1.49
Lys		6590 ± 712	3056		1.86 ± 0.20
Mb		3179 ± 1023	0		0.90 ± 0.29
Ova		4841 ± 634	1307		1.37 ± 0.18
C-Frag.		82526 ± 4444	78992	1.2	23.35 ± 1.26
BTNA pept.	1	4808 ± 654	1274	10	1.36 ± 0.19
	2	4747 ± 877	1213	5	1.34 ± 0.25
	3	5395 ± 1024	1861	20	1.53 ± 0.29
	4	5204 ± 828	1670	5	1.47 ± 0.23
	5	4398 ± 82	864	10	1.24 ± 0.02
	6	3262 ± 231	0	10	0.92 ± 0.07
	7	14454 ± 867	10920	5	4.09 ± 0.25
	8	8096 ± 1316	4562	40	2.29 ± 0.37
	9	5451 ± 77	1917	10	1.54 ± 0.02
	10	3160 ± 459	0	10	0.89 ± 0.13
	11	4020 ± 829	486	10	1.14 ± 0.23
	12	11141 ± 14	7607	40	3.15 ± 0.00
	13	7328 ± 165	3794	10	2.07 ± 0.05
	14	3575 ± 161	41	10	1.01 ± 0.05
	15	5204 ± 212	1670	10	1.47 ± 0.06
	16	3263 ± 256	0	10	0.92 ± 0.07
	17	6033 ± 1930	2499	10	1.71 ± 0.55
	18	4259 ± 674	725	10	1.21 ± 0.19
	19	7676 ± 1302	4142	10	2.17 ± 0.37
	20	3713 ± 881	179	20	1.05 ± 0.25
	21	26711 ± 2213	23177	40	7.56 ± 0.63
	22	4410 ± 400	876	20	1.25 ± 0.11
	23	5058 ± 628	1524	10	1.43 ± 0.18
	24	4738 ± 1233	1204	10	1.34 ± 0.35
	25	5723 ± 454	2189	40	1.62 ± 0.13
	26	3747 ± 299	213	20	1.06 ± 0.08
	27	3726 ± 208	192	10	1.05 ± 0.06
	28	2991 ± 221	0	10	0.85 ± 0.06
	29	4323 ± 644	789	5	1.22 ± 0.18
	30	3992 ± 808	458	10	1.13 ± 0.23
	31	6549 ± 395	3015	20	1.85 ± 0.11

Assay: 3/10/95. $5x10^5$ cells/well.

TABLE 2

Response to BoNt/A peptides 1-31 of LNC from **SJL** mice primed with 0.25 μg/mouse of C-Fragment of Botulinum Toxin

Challenge Antigen		CPM ± SD	Net CPM	Opt. Dose (μg/ml)	SI ± SD
Media		2330 ± 168	0		1.00 ± 0.07
Con A		39695 ± 6183	37365		17.04 ± 2.65
LPS		62828 ± 881	60498		26.96 ± 0.38
Lys		11260 ± 2298	8930		4.83 ± 0.99
Mb		2643 ± 730	313		1.13 ± 0.31
Ova		2692 ± 512	362		1.16 ± 0.22
Nonsense Pept.		4989 ± 793	2659	40	2.14 ± 0.34
C-Frag.		125096 ±14372	122766	5	53.69 ± 6.17
BTNA pept.	1	3219 ± 1077	889	40	1.38 ± 0.46
	2	5170 ± 783	2840	40	2.22 ± 0.34
	3	3888 ± 1412	1558	40	1.67 ± 0.61
	4	111343 ± 9325	109013	5	47.79 ± 4.00
	5	63318 ± 9251	60988	80	27.18 ± 3.97
	6	66581 ± 638	64251	80	28.58 ± 0.27
	7	84353 ± 3875	82023	2.5	36.20 ± 1.66
	8	89396 ±13932	87066	40	38.37 ± 5.98
	9	46336 ± 2251	44006	80	19.89 ± 0.97
	10	4463 ± 93	2133	10	1.92 ± 0.40
	11	7310 ± 755	4980	20	3.14 ± 0.32
	12	5284 ± 555	2954	10	2.27 ± 0.24
	13	3340 ± 471	1010	1.2	1.43 ± 0.20
	14	7163 ± 915	4833	40	3.07 ± 0.39
	15	23047 ± 67	20717	40	9.89 ± 0.03
	16	5170 ± 3184	2840	40	2.22 ± 1.37
	17	4335 ± 113	2005	2.5	1.86 ± 0.05
	18	2824 ± 383	494	10	1.21 ± 0.16
	19	6455 ± 1177	4125	40	2.77 ± 0.51
	20	8610 ± 2954	6280	40	3.70 ± 1.27
	21	4482 ± 142	2152	40	1.92 ± 0.06
	22	5703 ± 746	3373	20	2.45 ± 0.32
	23	3948 ± 1235	1618	20	1.69 ± 0.53
	24	3215 ± 749	885	0.6	1.38 ± 0.32
	25	2683 ± 178	353	20	1.15 ± 0.08
	26	3031 ± 734	701	20	1.30 ± 0.32
	27	1982 ± 234	0	10	0.85 ± 0.10
	28	1718 ± 324	0	0.6	0.73 ± 0.14
	29	2663 ± 153	333	10	1.14 ± 0.07
	30	2388 ± 987	58	40	1.02 ± 0.42
	31	3153 ± 402	823	10	1.35 ± 0.17

Assay: 1/3/95. $5x10^5$ cells/well.

FIGURE 1

Synthetic Peptides of *Clostridia botulinum* Toxin Type A

Peptide Number	Sequence Position	Structure
1	855-873	K Y V D N Q R L L S T F T E Y I K N I
2	869-887	Y I K N I I N T S I L N L R Y E S N H
3	883-901	Y E S N H L I D L S R Y A S K I N I G
4	897-915	K I N I G S K V N F D P I D K N Q I Q
5	911-929	K N Q I Q L F N L E S S K I E V I L K
6	925-943	E V I L K N A I V Y N S M Y E N F S T
7	939-957	E N F S T S F W I R I P K Y F N S I S
8	953-971	F N S I S L N N E Y T I I N C M E N N
9	967-985	C M E N N S G W K V S L N Y G E I I W
10	981-999	G E I I W T L Q D T Q E I K Q R V V F
11	995-1013	Q R V V F K Y S Q M I N I S D Y I N R
12	1009-1027	D Y I N R W I F V T I T N N R L N N S
13	1023-1041	R L N N S K I Y I N G R L I D Q K P I
14	1037-1055	D Q K P I S N L G N I H A S N N I M F
15	1051-1069	N N I M F K L D G C R D T H R Y I W I
16	1065-1083	R Y I W I K Y F N L F D K E L N E K E
17	1079-1097	L N E K E I K D L Y D N Q S N S G I L
18	1093-1111	N S G I L K D F W G D Y L Q Y D K P Y
19	1107-1125	Y D K P Y Y M L N L Y D P N K Y V D V
20	1121-1139	K Y V D V N N V G I R G Y M Y L K G P
21	1135-1153	Y L K G P R G S V M T T N I Y L N S S
22	1149-1167	Y L N S S L Y R G T K F I I K K Y A S
23	1163-1181	K K Y A S G N K D N I V R N N D R V Y
24	1177-1195	N D R V Y I N V V V K N K E Y R L A T
25	1191-1209	Y R L A T N A S Q A G V E K I L S A L
26	1205-1223	I L S A L E I P D V G N L S Q V V V M
27	1219-1237	Q V V V M K S K N D Q G I T N K C K M
28	1233-1251	N K C K M N L Q D N N G N D I G F I G
29	1247-1265	I G F I G F H Q F N N I A K L V A S N
30	1261-1279	L V A S N W Y N R Q I E R S S R T L G
31	1275-1295	S R T L G C S W E F I P V D D G W G E R P

FIGURE 2

Binding of BoNt/A-peptides to Human anti-BoNt/A antibody

(Net CPMs with Human IgG Net CPM also subtracted)

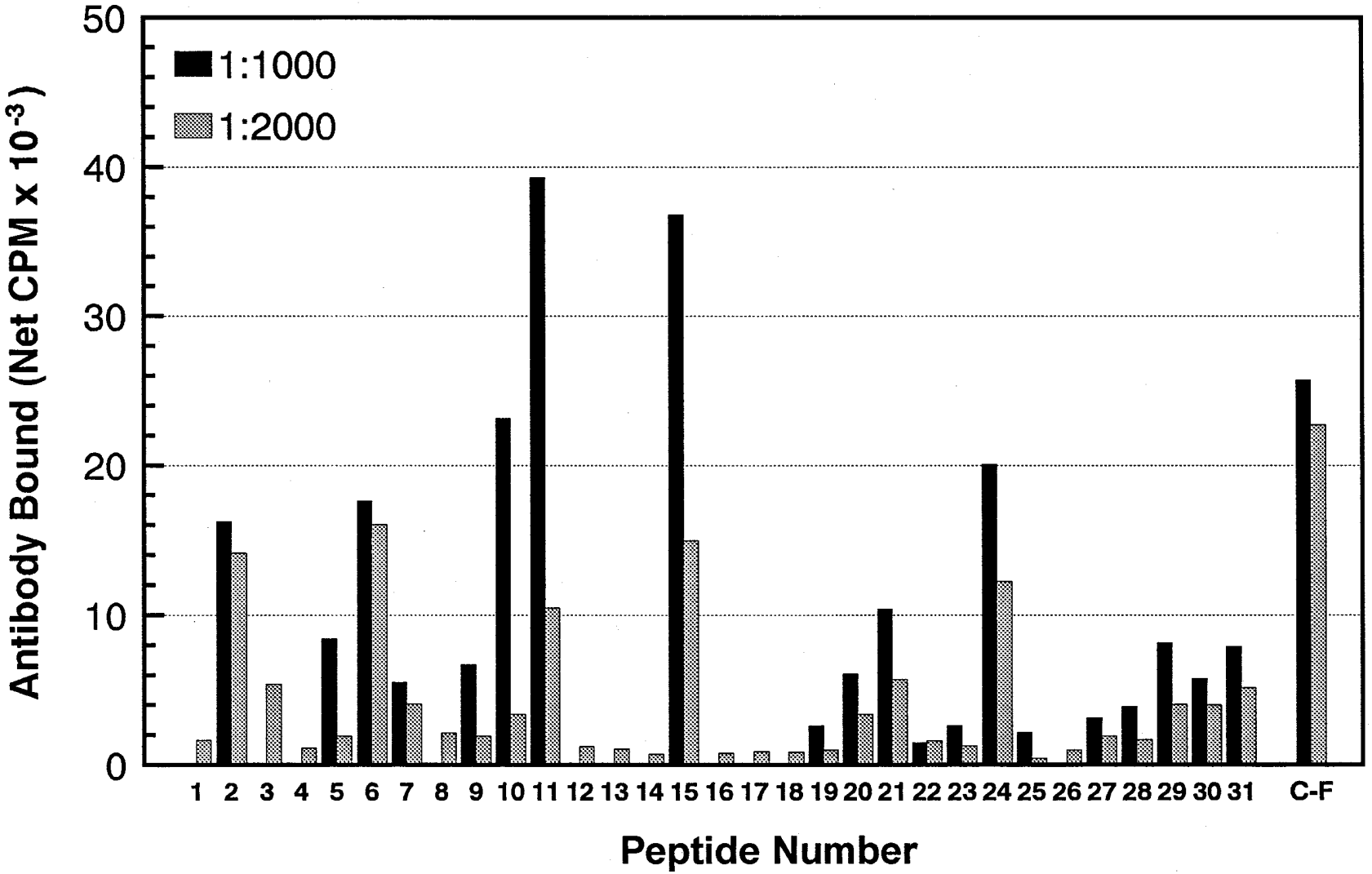


FIGURE 3

Binding of BoNt/A-peptides to Horse anti-BoNt/A antibody

(Net CPMs with Horse prebleed Net CPM also subtracted)

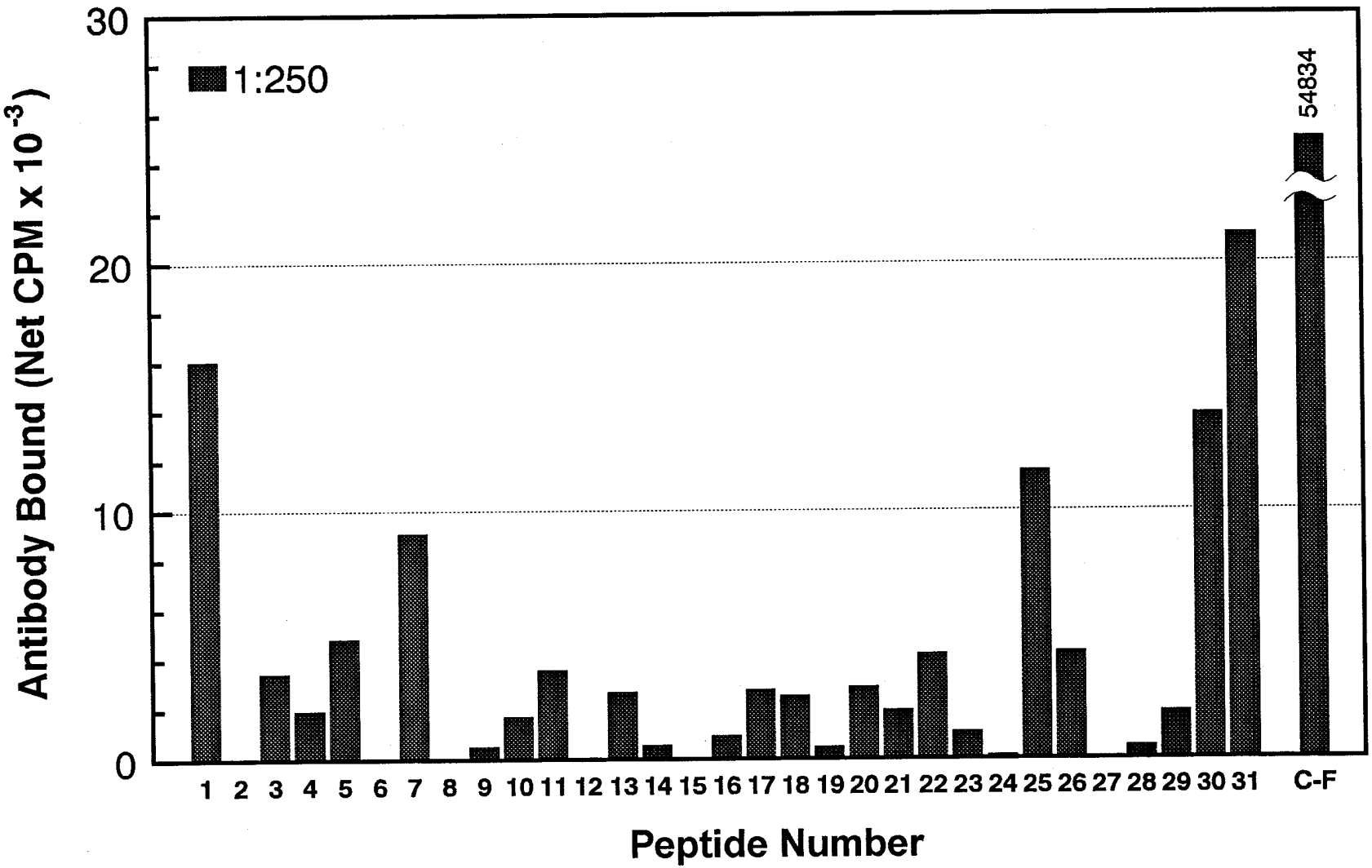
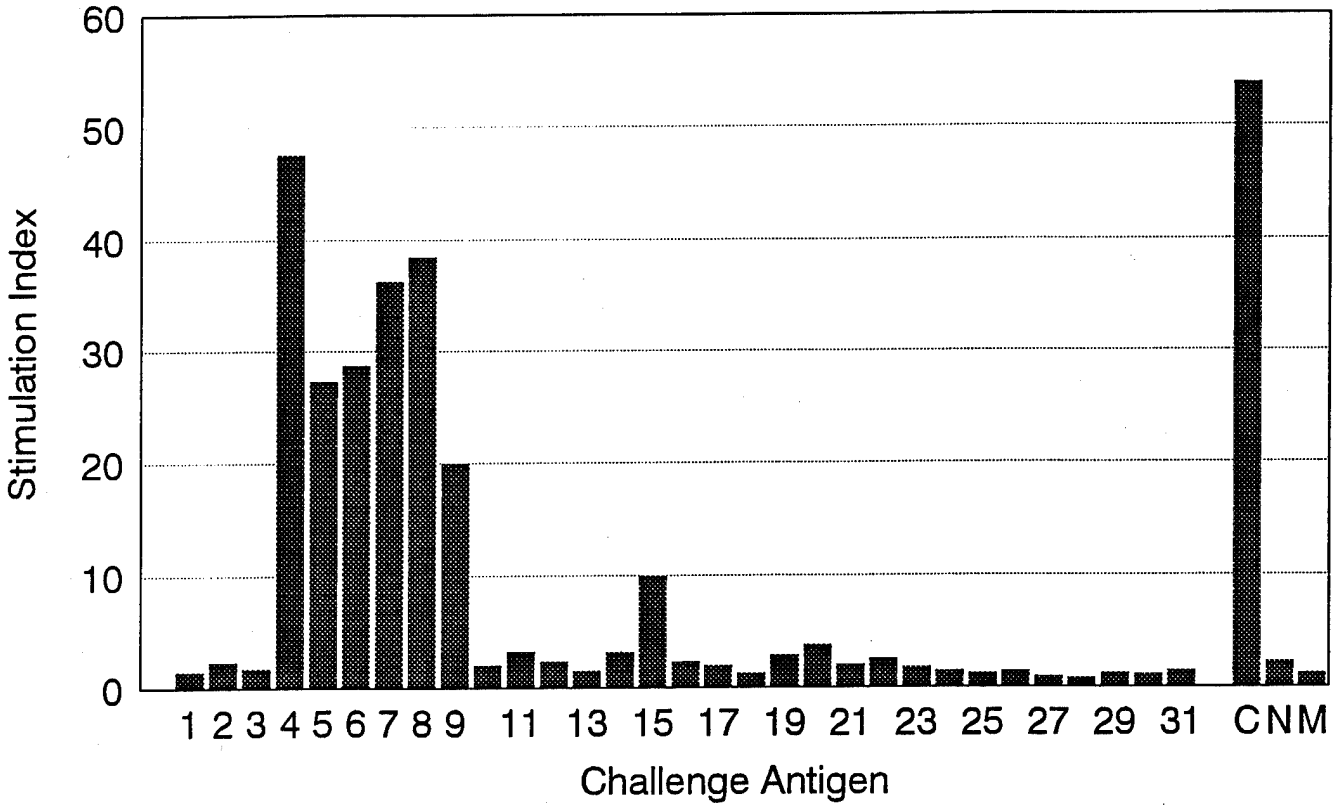


FIGURE 4

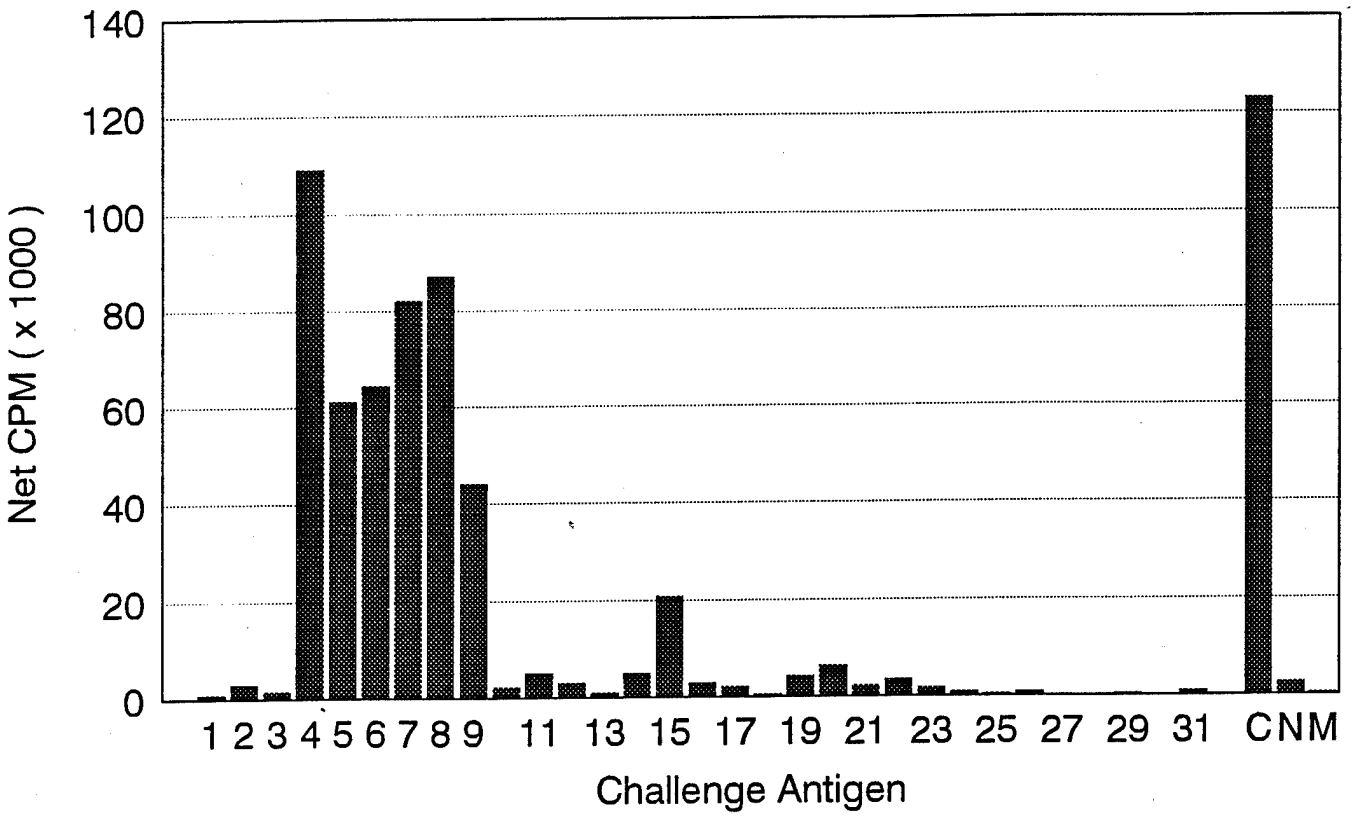
Proliferative response to BoNt/A peptides 1-31 of LNC from SJL primed with C-Fragment of Botulinum toxin



C, C-Frag.; N, Nonsense Pept.; M, Mb. Media= 2330 cpm. 5x05 cells/well, Assay: 1/3/95.

FIGURE 5

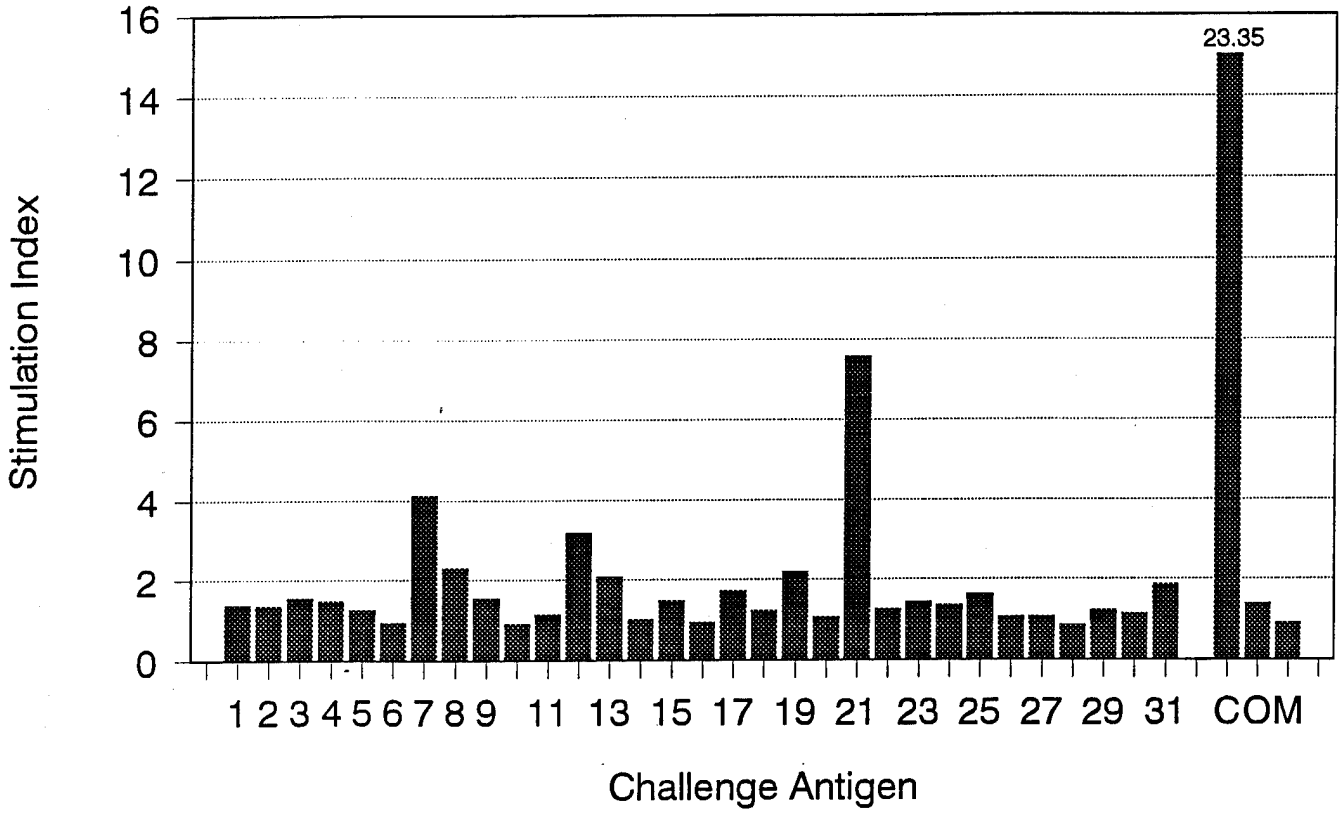
Proliferative response to BoNt/A peptides 1-31 of LNC from SJL primed with C-Fragment of Botulinum toxin



C, C-Frag.; N, Nonsense Pept.; M, Mb. Media= 2330 cpm. 5x05 cells/well, Assay: 1/3/95.

FIGURE 6

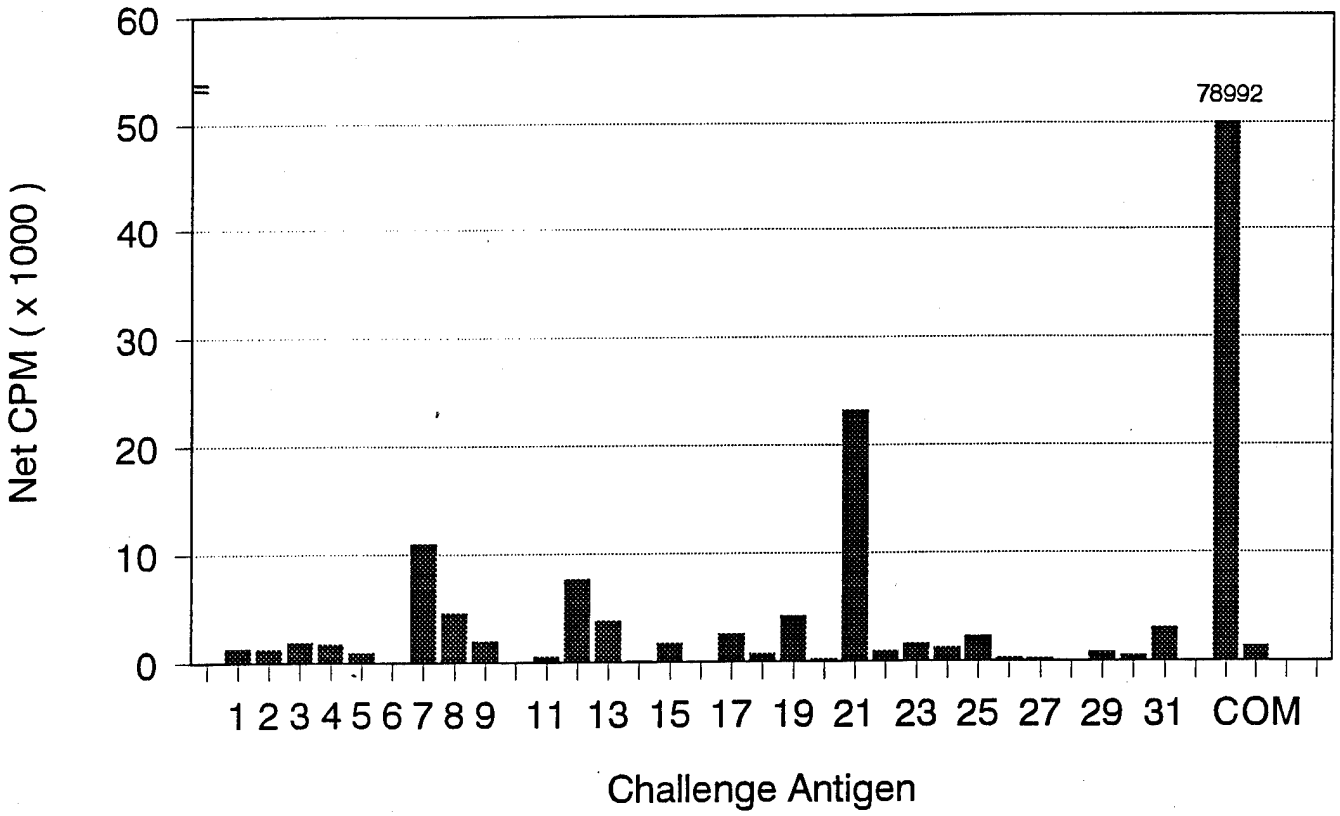
Proliferative response to BoNt/A peptides 1-31 of LNC from Balb/c primed with C-Fragment of Botulinum toxin



C, C-Frag.; O, Ovalbumin; M, Mb. Media= 3534 cpm. 5x05 cells/well, Assay: 3/10/95.

FIGURE 7

Proliferative response to BoNt/A peptides 1-31 of LNC from Balb/c primed with C-Fragment of Botulinum toxin



C, C-Frag.; O, Ovalbumin; M, Mb. Media= 3534 cpm. 5x05 cells/well, Assay: 3/10/95.